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PROTEIN STUDIES

Complete Amino Acid Sequence of Scenedesmus Ferredoxin

(H. Matsubara, K. Sugeno, D. Tsuchiya, and L. Nerenberg)

The amino acid sequence of *Scenedesmus* ferredoxin was completed. Chymotryptic and tryptic peptides from S-carboxymethylcysteinyl ferredoxin and oxidized ferredoxin were studied. Thermolysin and subtilisin were effectively used to degrade large peptides. Cyanogen bromide treatment of carboxymethylcysteinyl ferredoxin split the whole molecule into two portions and this provided the necessary means for arranging tryptic and chymotrptic peptides to complete independently the *Scenedesmus* ferredoxin sequence. The sequence is given in Fig. 1.

The amino acid composition deduced from the sequence agreed with that obtained from the analysis of the native ferredoxin. A total of 96 residues was present. The molecular weight was calculated to be approximately 10,350 including two atoms of non-heme iron and two moles of inorganic sulfide. Tryptophan and asparagine were absent. The sequence differed in 29 positions from that of spinach ferredoxin. One residue each of methionine, histidine, and arginine were present. The positions of one histidine (residue 90), one arginine (residue 40), three of four lysines (residue 4, 50 and 91), three of four prolines (residue 10, 19 and 36), five of six cysteines (residue 18, 39, 44, 47 and 77), six of seven glycines (residue 12, 32, 42, 49, 54 and 72), six of seven leucines (residue 7, 25, 35, 64, 75 and 95), all of four tyrosines (residue 3, 23, 37 and 80), two Asp-Asp groups (residue 20-21 and 65-66), and two Glu-Glu groups (residue 29-30 and 92-93) are invariant. They must have important roles in the maintenance of function on the protein molecule.

Partial Amino Acid Sequence of Chromatium Ferredoxin

(H. Matsubara, R. Sasaki and D. Tsuchiya)

The partial amino acid sequence of *Chromatium* ferredoxin was established. Thermolytic peptides from carboxymethylcysteinyl ferredoxin were overlapped by chymotryptic peptides of oxidized ferredoxin. The only portion of the sequence which was not established was that from residues 13 to 20. The sequence itself differed markedly from those of non-photosynthetic bacterial ferredoxins and of plant ferredoxins.

The distinctive features of *Chromatium* ferredoxin are as follows:

1. A total of 81 residues in contrast to 55 in the clostridial type.
2. The presence of methionine and histidine residues in contrast to the absence of these residues in clostridial type.
3. Extra sequences of nine residues, 42-50, and 17 residues, 65-81, which are absent from the clostridial type.

However, the most distinctive regions of the clostridial type structure were also in *Chromatium* ferredoxin, namely the distribution of cysteine residues and other invariant residues in all clostridial types as shown in Fig. 2.

Sequence Study of Taro Ferredoxin

(H. Matsubara, K. Rao and D. Tsuchiya)

The sequence of a ferredoxin isolated from one of the monocotyledonous plants, Hawaiian taro, is now under investigation. It is interesting to compare this ferredoxin with other plant ferredoxins. Nearly half the sequence has been completed.

Evolutionary Aspects of Ferredoxins

(H. Matsubara, T. H. Jukes and C. R. Cantor of Columbia University)

The known amino acid sequences of three clostridial types of ferredoxin, three chloroplast types of ferredoxin, and one photosynthetic bacterial type of ferredoxin were compared in terms of protein evolution. The repetitive sequences of clostridial type of ferredoxin suggested the

gene duplication of this protein in an early era of evolution. The most primitive ferredoxin seemed to be that of *Cl. butyricum*. By comparing the entire sequence of chloroplast type of ferredoxin with that of clostridial type ferredoxin, it was possible to postulate the process of evolution of ferredoxin (Fig. 3).

Furthermore, by comparing *Chromatium* ferredoxin with all other known ferredoxins, it was possible to postulate a phylogenetic tree of ferredoxins (Fig. 4).

Amino Acid Sequence of Pyridoxyl-Binding Site in Tryptophanase from E. coli

(H. Matsubara in collaboration with Drs. H. Kagamiyama and E. Snell of the Department of Biochemistry)

The tryptophanase from *E. coli* binds one pyridoxal phosphate making a Schiff's base per approximately 50,000 molecular weight of the protein which is one of the four identical subunits. Each subunit is composed of two chains whose identity is not completely known. The covalent bond of the Schiff's base is made by the reduction of the bond with sodium borohydride. The protein was digested by chymotrypsin and the fluorescent portion was purified. The structural study revealed the sequence to be Ser-Ala-Lys-Lys-Asp-Ala-Met-Val-Pro-Met.

pyridoxyl

It is interesting to note that there are two consecutive lysine residues and one neighboring aspartic acid in this peptide. They may play an important role in the mechanism of this enzyme.

Application of Tritiation Procedure in Obtaining the Carboxyl-Terminal Peptide of Proteins

(H. Matsubara, in collaboration with H. Matsuo, Department of Entomology)

Selective tritiation of the carboxyl-terminal amino acids in polypeptides has recently been proposed by H. Matsuo and his co-workers as a new method for identifying the carboxyl-terminal amino acids in proteins. This procedure was applied to obtain the carboxyl-terminal fragments of proteins.

The investigation was carried out with *Scenedesmus* ferredoxin and peptides derived from it during the course of structural studies. Figure 5 shows how tryptic digestion was conducted to identify the carboxyl-terminal fragment of Peptide C-V obtained from *Scenedesmus* ferredoxin. The results showed that the tritiated peptide underwent tryptic digestion smoothly and the carboxyl-terminal fragment was easy to detect by its radioactivity.

Scenedesmus ferredoxin was tritiated and the tritiated protein was directly digested by chymotrypsin. The radioactive spot on the peptide map of the digest was easily detected in the scintillation spectrophotometer and the composition of this spot agreed well with the theoretical values for the carboxyl-terminal peptide of *Scenedesmus* ferredoxin.

Future Plans

1. Characterization of the reactivity of SH groups in *Scenedesmus* ferredoxin.
2. Chemical and enzymic modification of plant type ferredoxin.
3. Sequence study of taro ferredoxin.
4. Sequence study of tryptophanase (in collaboration with Dr. Snell's laboratory).

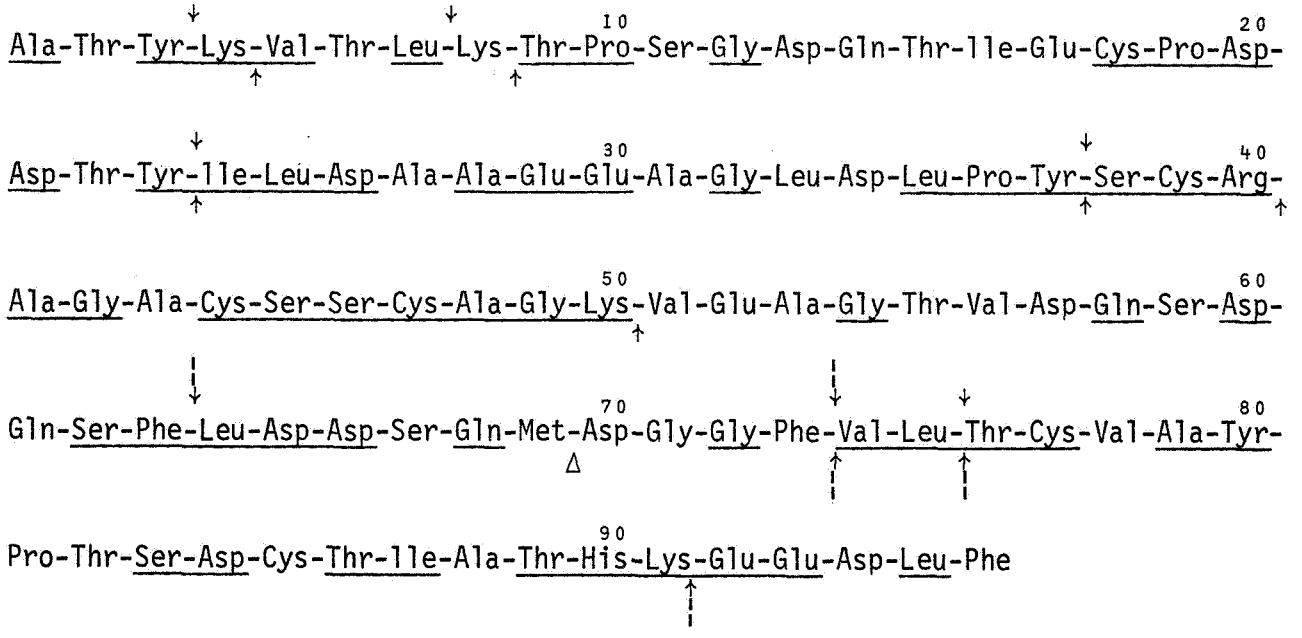

Ala-Thr-Tyr-Lys-Val-Thr-Leu-Lys-Thr-Pro-Ser-Gly-Asp-Gln-Thr-Ile-Glu-Cys-Pro-Asp-
Asp-Thr-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-Ala-Gly-Leu-Asp-Leu-Pro-Tyr-Ser-Cys-Arg-
Ala-Gly-Ala-Cys-Ser-Ser-Cys-Ala-Gly-Lys-Val-Glu-Ala-Gly-Thr-Val-Asp-Gln-Ser-Asp-
Gln-Ser-Phe-Leu-Asp-Asp-Ser-Gln-Met-Asp-Gly-Gly-Phe-Val-Leu-Thr-Cys-Val-Ala-Tyr-
Pro-Thr-Ser-Asp-Cys-Thr-Ile-Ala-Thr-His-Lys-Glu-Glu-Asp-Leu-Phe
10
20
30
40
50
60
70
80
90
Δ

Figure 1

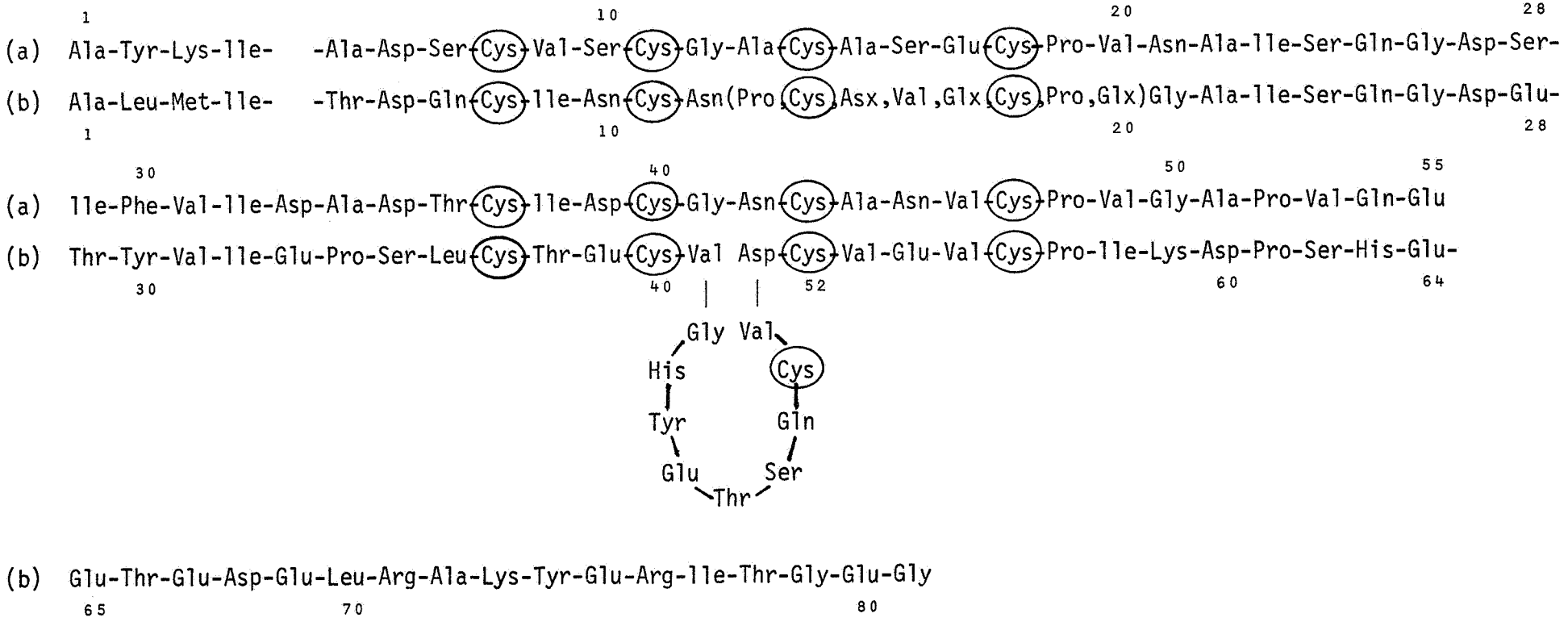
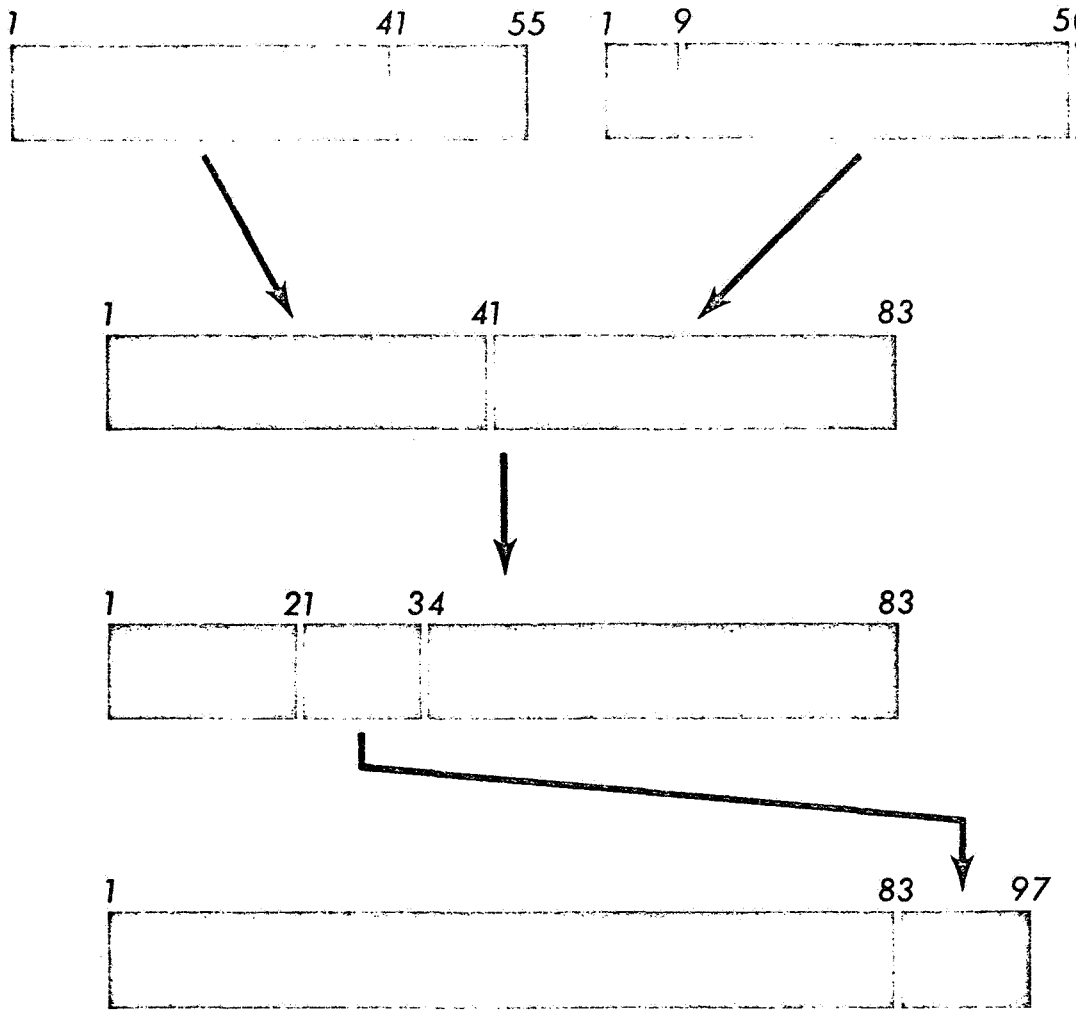

1
10
20
28
(a) Ala-Tyr-Lys-Ile- -Ala-Asp-Ser-Cys-Val-Ser-Cys-Gly-Ala-Cys-Ala-Ser-Glu-Cys-Pro-Val-Asn-Ala-Ile-Ser-Gln-Gly-Asp-Ser-
(b) Ala-Leu-Met-Ile- -Thr-Asp-Gln-Cys-Ile-Asn-Cys-Asn(Pro,Cys,Asx,Val,Glx,Cys,Pro,Glx)Gly-Ala-Ile-Ser-Gln-Gly-Asp-Glu-
1
10
20
28
30
40
50
55
(a) Ile-Phe-Val-Ile-Asp-Ala-Asp-Thr-Cys-Ile-Asp-Cys-Gly-Asn-Cys-Ala-Asn-Val-Cys-Pro-Val-Gly-Ala-Pro-Val-Gln-Glu
(b) Thr-Tyr-Val-Ile-Glu-Pro-Ser-Leu-Cys-Thr-Glu-Cys-Val Asp-Cys-Val-Glu-Val-Cys-Pro-Ile-Lys-Asp-Pro-Ser-His-Glu-
30
40
52
60
64
Gly Val
His
Cys
Tyr
Gln
Glu
Ser
Thr
(b) Glu-Thr-Glu-Asp-Glu-Leu-Arg-Ala-Lys-Tyr-Glu-Arg-Ile-Thr-Gly-Glu-Gly
65
70
80

FIGURE 2



Portions of two bacterial ferredoxins

join to form a longer molecule

which differentiates and lengthens by duplication

of a central region which is translocated to the end to form plant ferredoxin.

Evolution of Chloroplast-Type Ferredoxin

FIGURE 3

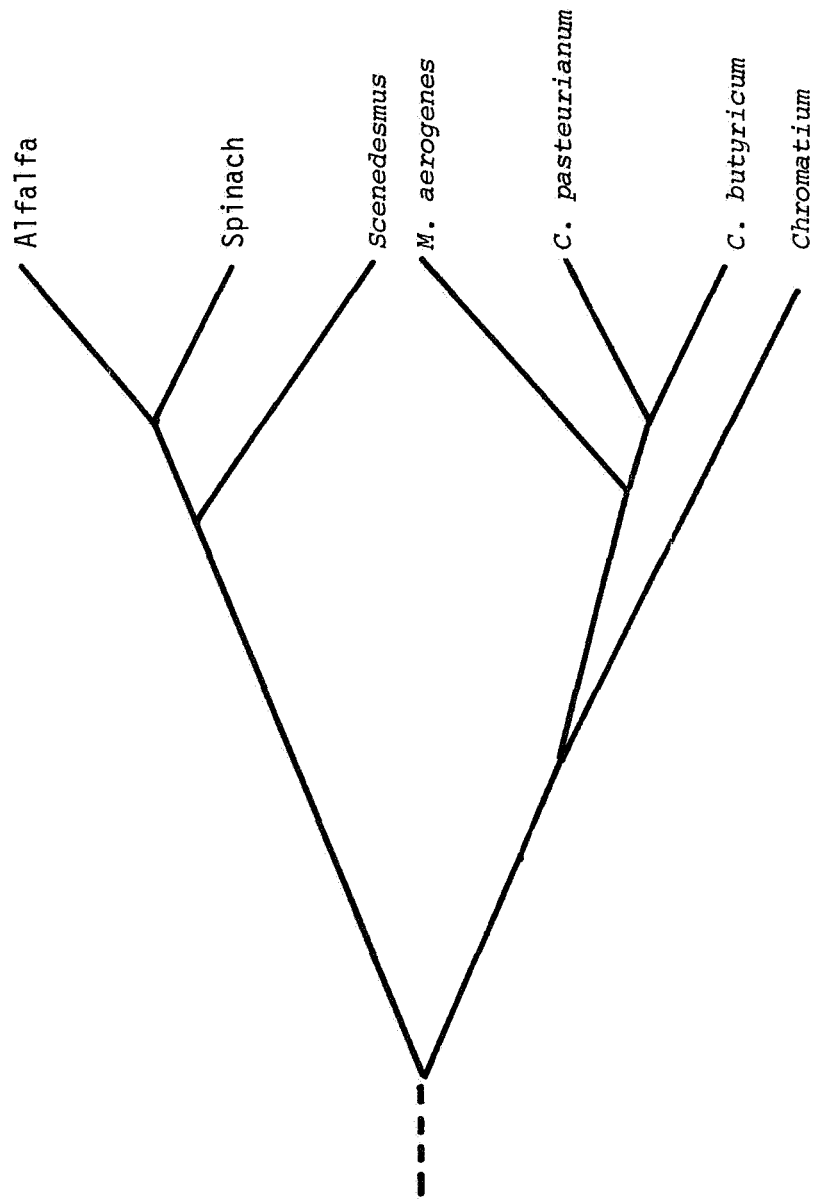

Alfalfa
Spinach
Scenedesmus
M. aerogenes
C. pasteurianum
C. butyricum
Chromatium

FIGURE 4

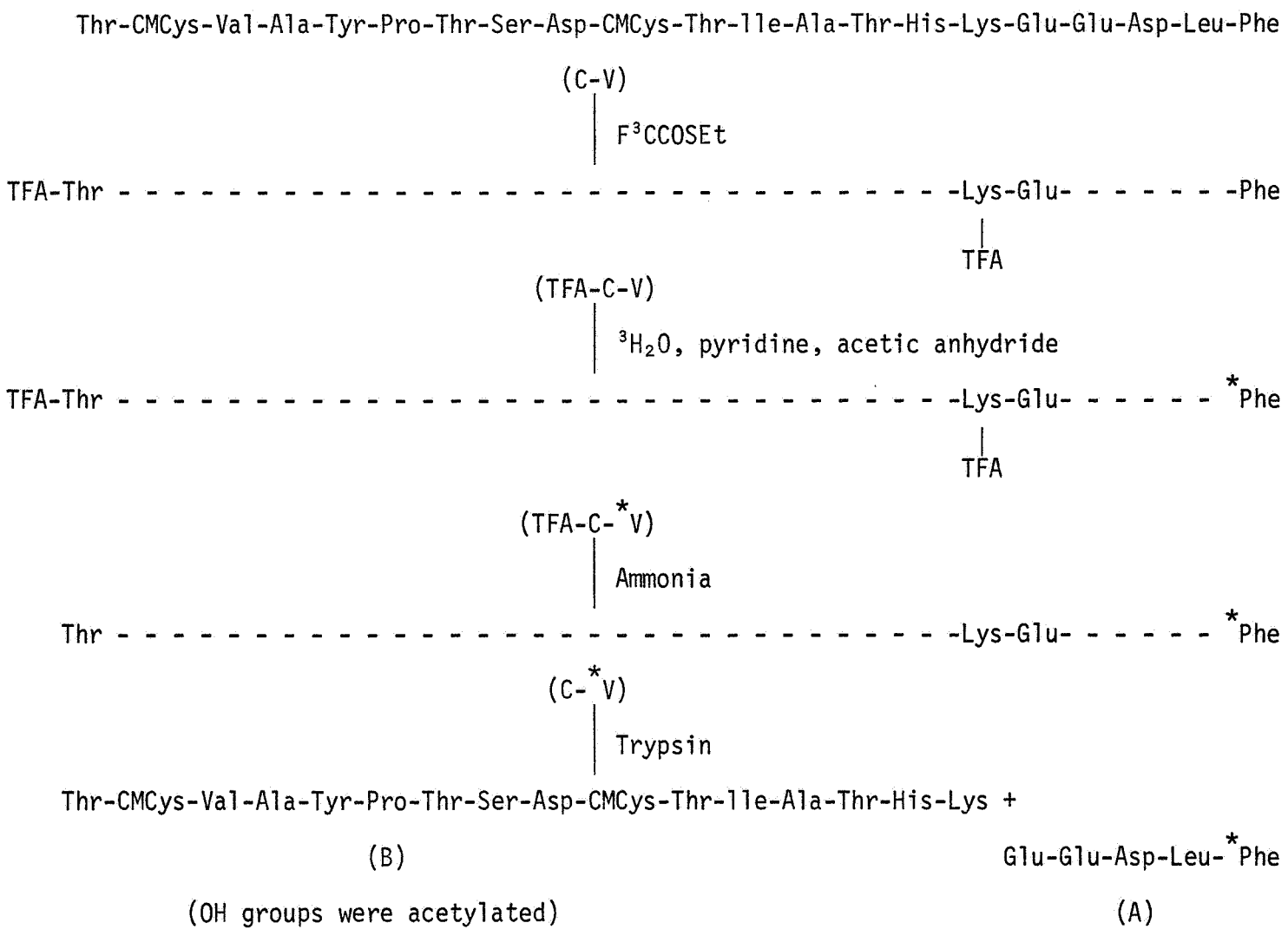

Thr-CMCys-Val-Ala-Tyr-Pro-Thr-Ser-Asp-CMCys-Thr-Ile-Ala-Thr-His-Lys-Glu-Glu-Asp-Leu-Phe
(C-V)
F3CCOSEt
TFA-Thr -Lys-Glu- - - - - - -Phe
TFA
(TFA-C-V)
3H2O, pyridine, acetic anhydride
TFA-Thr -Lys-Glu- - - - - - *Phe
TFA
(TFA-C-*V)
Ammonia
Thr -Lys-Glu- - - - - - *Phe
(C-*V)
Trypsin
Thr-CMCys-Val-Ala-Tyr-Pro-Thr-Ser-Asp-CMCys-Thr-Ile-Ala-Thr-His-Lys +
(B)
Glu-Glu-Asp-Leu-*Phe
(OH groups were acetylated)
(A)

FIGURE 5

OPTICAL PROPERTIES OF SUPERCOILED DNA MOLECULES

(M. F. Maestre, K. Sieux and C. Hurlbut Space Sciences Laboratory and J. Wang Chemistry Department)

The ORD spectra of supercoiled molecules that have covalently bonded in a supercoiled state were studied as a function of the number of superturns per unit length of the DNA molecule. The difference spectra of these supercoiled molecules shows a linear relationship with the number of superturns. This relationship is given by the following equation:

$$N/L = -4.55\ [\Delta\ \alpha]_{minimum}$$

where N = number of turns

L = length of the DNA molecule in μ

$[\Delta\ \alpha]_{minimum}$ = is the specific rotation at the trough of the difference ORD spectra.

Assuming the above formula to hold true for the state of the DNA molecule in such structures as viruses, it is possible to classify virus structures in terms of possible coiled DNA structures in their protein capsids or heads in the case of bacteriophages. Keeping in mind that the above equation is only a first order approximation to the very complicated relationship between geometrical structure and optical properties the following numbers of superturns can be assigned to the bacteriophages whose ORD spectra have been obtained.

Bacteriophage	Total number of superturns
T2	690
T4	795
T6	860
T7	63
λ	102
T5	160
α	56

A refinement of this technique could possible elucidate in more detail the internal structure of the viral DNA and perhaps could be extended to the study of more complex nucleoproteins such as chromosomes.

Circular Dichroism Studies

(M. Maestre and M. J. Tunis)

During the past year, we have been studying the circular dichroism of DNA films. The circular dichroism and other optical properties of DNA in solution could provide a sensitive index to its structure; but these properties are not well understood theoretically and the spectra are difficult to interpret. The structure of DNA in the solid form under various conditions of relative humidity and salt control is known from x-ray diffraction experiments. If the optical properties could be measured for these known structures, then this knowledge would be useful for correlating spectra with structure in solution.

We have succeeded in obtaining circular dichroism spectra of DNA films under conditions similar to those under which x-ray diffraction shows the existence of B, A and C structures. The spectra of these three forms of DNA differ considerably from each other: the presumed B form looks like the normal spectrum of DNA in solution; the A form resembles the circular dichroism of RNA; and the spectrum of the C form is like that of DNA in the presence of high salt concentrations.

The measured circular dichroism of films is very sensitive to any anisotropy in the film. We have derived a formula expressing the effect of orientation on the measured circular dichroism of the films. Using this equation, we can test for anisotropy and correct for small orientation effects.

References

1. Maestre, M. F. and Tinoco, Jr., I. (1967). *J. Mol. Biol., 23,* 323.
2. M. J. Tunis and J. B. Hearst (1968). *Biopolymers, 6,* 1218.

BASE SEQUENCES IN RNA

(S. Mandeles, D. Garfin, F. Fearney, C. Meier, L. Johnston, L. Wong, and R. Anderson)

Progress in the determination of base sequences in nucleic acids has continued along the lines that have been recently developed. These lines are: the use of the phenomenon of stripping to locate unique sequences in TMV RNA, and the development of methods for the formation of specific subunits from ribosomal RNA.

Stripping Experiments

Most of the work on the use of partially stripped TMV to locate unique sequences has been centered on attempts to improve the precision. The effect of changes in concentration, pH, and temperature on the distribution of particle sizes in partially stripped TMV is being studied, together with more rapid and efficient ways to control the extent of stripping (Lily Wong). The actual distribution of particle sizes obtained after the stripping reaction is being studied by electron microscopy (Mitchell Berman) and this is being correlated with electric birefringence measurements (Dr. George Sheats) in an attempt to develop a rapid and accurate method for determining the extent of the stripping reaction as well as the size distribution of the stripped particles.

Experiments are also under way to determine which oligonucleotides are produced when as little as 1% of the nucleic acid is exposed to nuclease action, and if oligonucleotides as small as tetramers can be used in mapping. This latter approach depends on the fact that a tetramer such as CpCpCpGp will probably occur only six times in a molecule of TMV RNA. A very precise method for mapping sequences will permit an accurate location of tetramers or larger non-unique oligomers in TMV RNA.

A gel electrophoresis method has been developed that separates oligonucleotides in the range of 10 to 100 nucleotides chain length. Electrophoretic separations of T1 RNase digests of TMV RNA have been carried out on as little as 100 micrograms of total digest. The unique oligomers Ω, ψ_1 and ψ_2 are well separated from the shorter oligomers and easily visible. This procedure is meant to serve as a basis for the analysis of unique oligonucleotides from very small amounts of hydrolysate in the stripping experiments (Lon Johnston).

A study has been made of the various methods for the hydrolysis of nucleic acid to yield oligonucleotides with terminal C only (Richard Anderson). The use of a carbodiimide derivative to block the uridine residues of TMV RNA was not completely successful, due on the one hand to the incomplete reaction of the carbodiimide with the uridine residues, and on the other to the incomplete removal of the blocking group after the digestion with pancreatic nuclease has taken place. The results with a

cytosine specific RNase (C-RNase) isolated from commercial bacterial alkaline phosphatase were a little more encouraging. Analysis of a C-RNase digestion of TMV RNA revealed a fairly specific although probably not complete digestion of TMV RNA to yield oligonucleotides with C terminals. This may have been due to insufficient enzyme or to some as yet unknown higher order of specificity.

Ribosomal RNA Subunits

(in collaboration with Drs. K. Hosokawa and M. A. Q. Siddiqui)

The RNA from 30s ribosomes (16s RNA), 50s ribosomes (23s RNA) and TMV were each digested separately with T1 and pancreatic RNase. The products of the digestion were separated on DEAE-Sephadex chromatographically according to chain length and estimates were made of the chain lengths of the various fractions (Colleen Meier). The pattern from the T1 digestion of TMV RNA served as a standard. It was found that when the log of the charge of an oligomer is plotted against the ionic strength of the eluting solvent, a straight line is obtained for oligonucleotides up through the chain length of twelve, after which the relationship is not easily discerned on the chromatographic patterns. Furthermore, if, in this same pattern, the log of the charge of the omega-mer (71+) and the psi-mers (27+) are plotted versus ionic strength, a second straight line can be drawn that intersects the first line at about a chain length of 15. This implies that the chromatographic column is behaving like two different columns and that this behavior is related to the size of the oligonucleotides. This observation is compatible with the fact that DEAE-Sephadex works both by ion-exchange and molecular size exclusion.

The patterns of the other digests revealed that no oligonucleotides greater than a chain length of 11 were produced from the action of T1 RNase on 16s or 23s RNA, or the action of pancreatic RNase on TMV RNA. However, the patterns from the pancreatic RNase digestion of 16s and 23s RNA each showed a fraction well separated from the body of the main pattern. The positions of the fraction in both patterns were the same and indicated an average chain length of about 35 nucleotides. The shape of the fraction indicated that more than one component was present, but attempts to separate this into individual oligonucleotides have not

yet been successful. The oligonucleotides in these fractions or core material presumably contain long sequences of A and G terminated with a pyrimidine. Preliminary measurements indicate that there is an equivalent amount of core in each of the 16s and 23s RNAs. The purpose of these experiments is to obtain easily characterized and possibly unique oligonucleotides from ribosomal RNA that can be used in studies of ribosomal subunits.

Future Plans

1. Work will continue on the stripping reaction to
   a) Increase the precision of the location of unique as well as smaller oligonucleotide sequences
   b) Determine the oligonucleotides that are produced when only very short lengths of RNA are exposed
   c) Develop rapid micromethods for oligonucleotide separations for use in stripping experiments.
2. Experiments on the formation of ribosomal RNA subunits will enter the next stage: ribosomal particles will be treated with RNase to produce large fragments. The RNA from these large fragments will be tested for the presence of core material. If the core material is partly or wholly destroyed after brief treatment of the ribosome with nuclease, this indicates that the core sequence is partly or wholly exposed on the surface of the ribosome. If the core material remains intact this would indicate that its sequences are in the interior of the ribosome. In this latter case, the core material can be used to identify and help characterize specific ribosomal subunits.

STUDIES WITH THE *A. VINELANDII* RNA POLYMERASE

(J. Krakow, J. W. Daley, E. Fronk, M. Karstadt)

The rIC Copolymer

RNA polymerase, incubated with ITP, CTP and Mn^{++} in an unprimed interaction synthesizes, after a lag phase, a copolymer of alternating IMP and CMP residues (Krakow, Karstadt, *PNAS, 58*, 2094, 1967). Similar

reactions with GTP and CTP show no product. Recently, we have found that rIC will serve as a template for either rIC or rGC synthesis. When rGC was used as a template, no synthesis of rGC was obtained thereby providing an explanation for the failure to obtain unprimed polymer synthesis with GTP and CTP. The T_M for the rIC copolymer is 47° in 0.1 M sodium phosphate, pH 7.5 and for rGC the T_M is 87° in 0.001 M sodium phosphate, pH 7.5. Both alternating copolymers melt at a lower temperature than the corresponding homopolymer pairs (rI:rC; rG:rC).

We have been using acrylamide gel electrophoresis as a convenient and sensitive method for analyzing RNA polymerase monomer, dimer forms as well as template enzyme complexes. This has been facilitated by a method devised in our laboratory for assaying for polymerase activity in the gel matrix (Krakow, Daley and Fronk, *BBRC, 32*, 98, 1968).

Release of the γ Protein from Template-Enzyme Complexes

We have found that upon formation of template enzyme complexes, there is a concomitant release of a protein component (γ protein) which apparently forms part of the dimer complex. This is best seen on dissociation of polymerase by tRNA which produces monomer-tRNA complex + the γ protein which has a rapid electrophoretic mobility. When the tRNA-monomer complex is treated with ribonuclease to degrade the tRNA, the dimer reforms and the γ protein band disappears, presumably due to its reinsertion into the dimer complex. The γ protein is displaced by denatured DNA, poly U and rIC copolymer, but not heparin or dextran sulfate both of which dissociate the enzyme into polyanion-monomer complexes.

Enzyme Transitions During Unprimed rIC Synthesis

We have found that at a period corresponding to the end of the lag phase, the normal dimer-monomer pattern is markedly altered. At this time, a new form of the enzyme appears tentatively considered a tetramer in which the newly-formed rIC hold 2 dimer molecules together. At later times during active rIC synthesis, the tetramer, dimer and free monomer bands are missing and the gels show a smeared pattern terminating at the position occupied by the rIC-monomer species. The γ protein has also

been displaced since all enzyme is bound to template. On pretreatment with T_1-RNase, the smear resolves to a predominantly rIC-monomer band. This supports the idea that the form of the enzyme engaged in transcription is the monomer and not the dimer.

Future Plans

1. We plan to further characterize the rIC directed synthesis of rIC and rGC. To this end, we have recently obtained the dIC copolymer and plan to compare dIC and rIC directed reactions to see if there are any differences in the kinetics resulting from these templates.

2. We have to purify the γ protein and determine its molecular weight and what role it plays in the structure of the polymerase dimer. We will prepare tRNA-monomer (lacking the γ protein) and study its synthetic and binding abilities. We also plan to determine what the subunit structure of the tRNA monomer is compared to the dimer.

3. We have initiated a series of experiments in the mechanism of inhibition of polymerase by the antibiotic streptovaricin. This is known to inhibit the enzyme by binding to the protein and not to the template (i.e., actinomycin).

ACTION OF VARIOUS MUTAGENS ON TMV AND TMV-RNA

(B. Singer [Mrs. H. Fraenkel-Conrat], in collaboration with the Department of Molecular Biology)

Since we have now found that nitrosoguanidine (MNNG) alkylates guanine, adenine, and cytosine in nucleic acids, it was of interest to compare the mechanism and mutagenic effect of this alkylation with other alkylating agents (dimethyl sulfate (DMS), diethyl sulfate, methyl methanesulfonate, ethyl methanesulfonate, N-nitroso-N-methyl urethane, etc.).

Earlier work from this laboratory suggested that methylation of TMV-RNA was poorly mutagenic, while introduction of larger alkyl groups was not mutagenic. MNNG is also a poor mutagen for TMV-RNA, but a very good mutagen for the RNA in the intact virus. In addition, only MNNG (of the alkylating agents studied) was unable to alkylate bases, nucleotides, or dinucleotides, and required a much longer chain length for reaction. While the usual sites of alkylation (the 7 position of G, 1 position of A, and 3 position of C)

were reactive to MNNG, other still-unidentified products were found, the most striking of which was a fluorescent methylated nucleotide tentatively assumed to be a derivative of adenine.

The work now in progress comparing the extent and type of alkylation with the mutagenicity is summarized in Tables I and II.

The most noticeable reaction difference correlated with mutagenicity is that the relative amount of 3-methyl C produced by MNNG is dramatically increased in the virus which then has a high mutagenicity, whereas DMS treatment of virus is not mutagenic and no detectable 3-methyl C is found. A corollary to this is that DMS is a better mutagen for RNA than MNNG and in this case, more 3-methyl C is found after DMS than MNNG. This correlation is by no means established and further work on identifying relatively minor products of alkylation is in progress. In addition, work is also being undertaken to explore the possibility that MNNG causes deamination of adenine in the virus and this is the mutagenic event.

Future Plans

Work will continue on the comparative study of the reaction of dimethyl sulfate of other methylating agents and nitrosoguanidine on single-stranded polymers, double-stranded polymers, RNA and TMV in an effort to ascertain which, if any, base alkylating is mutagenic.

The effect of conformation of polynucleotides on the relative alkylation of the N-1, N-3, and N-7 of adenine, the N-7 of guanine, and the N-3 of cytosine is also being studied.

TABLE I

RELATIVE QUANTITIES OF METHYL DERIVATIVES

AFTER REACTION WITH MNNG AND DMS

	Alkylating Agent	Homopolymer % of orig. OD	TMV-RNA % of orig. OD	TMV % of methyl-ation*
7-Methyl G	MMNG		20 — 40	70
	DMS		20	94
1-Methyl A	MNNG	12	7	1
	DMS	40 — 50	5	4
3-Methyl C	MNNG	< 1**	<< 5**	26
	DMS	50 — 60	4	< 1
Fluorescent A	MNNG		2 — 4	2
	DMS		0	< 0.05

* MNNG alkylates 2 — 3 bases in virus, while over 500 bases are alkylated by DMS.

**When the reaction is performed in dimethyl formamide, instead of water, about 10% of the poly C is methylated. The figures given are the limit of detection with the methods used.

TABLE II

MUTAGENICITY OF ALKYLATING AGENTS ACTING ON TMV-RNA AND TMV*

Mutagen	TMV-RNA	TMV
Nitrosoguanidine	2	23
Dimethyl Sulfate	5	1
Diethyl Sulfate	1	1
Methyl Methanesulfonate	4	1
Ethyl Methanesulfonate	1	—
Nitrous Acid**	34	110

* Mutagenicity is defined as the ratio of local lesions found with the treated RNA or virus to the control (spontaneous or endogeneous mutants) when applied to *N. sylvestris* at equal concentrations of infective particles.

**These data are included for reference only.

BIOSYNTHESIS OF THE INOSINIC ACID OF TRANSFER RNA (t-RNA)

(H. Kammen, S. Spengler)

Inosinic acid is a normal constituent of yeast and *E. coli* t-RNA. The inosinic acid residues are localized in the "anti-codon loop" of specific t-RNA molecules, and perform a major function in codon recognition during protein synthesis.

The biosynthesis of these inosinic acid residues must occur by one of two mechanisms (1) by introduction into RNA chains during polynucleotide assembly by RNA polymerase; (2) by enzymatic modification of an appropriate polynucleotide precursor of t-RNA. These possibilities should be distinguishable from the labeling of the purine nucleotide of t-RNA after the growth of cells with isotopic purine precursors. Mechanism (1) requires that the labeling of the inosinic acid residues should be dependent upon, and should reflect changes in the labeling of acid-soluble inosine nucleotides, especially ITP, whereas mechanism (2) predicts the opposite.

A critical test of these alternatives has been carried out with *E. coli* strain B-94 as the test organism. This mutant was chosen for its inability to convert hypoxanthine to adenine nucleotides, a metabolic defect which permits the preferential dilution of inosine nucleotides to be achieved without a parallel dilution of adenosine nucleotides. The results of the experiment (Table I) show that the conversion of ^{14}C-adenine to the inosinic acid of t-RNA is not altered by conditions which dilute the inosine nucleotide pools. (The labeling of the guanylic acid of t-RNA serves as an internal indicator of the success of the dilution.) These results rule out mechanism (1), above, and point to a polynucleotide modification as the mechanism for the synthesis of the inosinic acid residues. The most likely modification reaction would be a deamination reaction, a probability which is supported by the constant adenylic acid/inosinic acid ratio in the t-RNA.

RNA polymerase does not discriminate between GTP and ITP in their base-pairing properties. Since the insertion of inosinic acid residues in place of guanylic acid residues would lead to lethal consequences, *E. coli* (and other organisms) must prevent the access of RNA polymerase to ITP.

We have attempted to measure the size of the ITP pool of *E. coli* by ^{32}P-labeling techniques, but have been unable to detect any significant amounts of this metabolite (less than 0.3% of the amount of ATP).

Accordingly, the behavior of crude extracts of *E. coli* was examined for their ability to phosphorylate a variety of nucleoside mono- and diphosphates, and to dephosphorylate triphosphates. Such preparations readily converted IDP to ITP, and did not show any preference for the degradation of ITP. However, these extracts were totally incapable of converting IMP to IDP. The surprising absence of this reaction would appear to be the primary means for restricting the production of ITP in *E. coli*.

TABLE I

INCORPORATION OF ^{14}C-ADENINE

INTO PURINE NUCLEOTIDES OF *E. coli* B-94 t-RNA

Nucleotide Fraction	^{14}C Incorporated, c.p.m.	
	Minus Hypoxanthine	Plus Hypoxanthine
Adenylic acid	67,800	77,220
Guanylic acid	113,570	23,510
Inosinic acid (after ion-exchange and thin-layer chromatography)	442	536
Inosinic acid (after ion-exchange chromatography, enzymic dephosphorylation, and recovery of inosine by thin-layer chromatography)	420	487
cpm adenylic acid/cpm inosinic acid	153	144
cpm guanylic acid/cpm inosinic acid	266	43.9

REGULATION OF NUCLEOSIDE METABOLISM IN *E. coli*

(H. Kammen, R. Koo)

Properties of Phosphodeoxyribomutase

We have continued the study of this enzyme, purified from *E. coli* B and from strain B-68 (which lacks deoxyribose-5-phosphate aldolase). The deoxyribomutase is readily distinguishable from *E. coli* phosphoglucomutase by its reaction requirements, by its mode of fractionation, and by its physical properties. Zone sedimentation in sucrose indicates a molecular weight of approximately 41,000 for the deoxyribomutase (vs 62,000 for the glucomutase).

The substrate specificity has been examined by a competition assay, which has been necessitated by the presence of residual phosphoglucomutase in our best preparations. Sugar phosphates which act as substrates for the enzyme should be competitive inhibitors of deoxyribose-1-phosphate (although competitive inhibitors need not be substrates). Compounds which act non-competitively, or which do not inhibit would probably not be substrates. Of a series of compounds tested, the only competitive inhibitors of deoxyribose-1-phosphate found included α-D-deoxyribose-5-phosphate, -ribose-1-phosphate, -ribose-5-phosphate and -xylose-1-phosphate. The corresponding α-D-glucose-1- and -mannose-1-phosphates are strongly inhibitory, but are non-competitive with deoxyribose-1-phosphate; α-D-fructose-1- and -galactose-1-phosphates are very weak inhibitors. These findings lend support to our earlier proposal, based on the behavior of mutant strains, that the specificity of the enzyme is not limited to deoxyribose phosphates, but may extend to a larger group of pentose phosphates. Final verification of this possibility will require direct demonstration of the interconversion of other pentose phosphates.

From these and other experiments, the enzyme appears to recognize the chain length of the sugar moiety, and the α-configuration at C_1, but does not distinguish between H- and OH and C_2 of the sugar, or the configuration of the OH group at C_3.

The mutase is highly sensitive to inhibition by phosphate compounds, notably those containing pyrophosphate groups. The order of inhibitory action is: nucleoside triphosphates or PP_i > nucleoside diphosphates and

P_i >> nucleoside monophosphates or nucleosides. The pyrophosphate inhibitors are non-competitive with respect to deoxyribose-1-phosphate, and the effects of nucleotide inhibitors are due solely to the phosphate groups. There are no essential differences which can be ascribed to the type of base or the type of sugar in the nucleotide.

At equilibrium, the reaction lies far in the direction of pentose-5-phosphate formation (approximately 95% conversion of deoxyribose-1-phosphate). The reverse reaction, the formation of doxyribose-1-phosphate from the -5-phosphate, has been observed, but the reaction is very slow, as might be expected from the equilibrium behavior. These features make it highly unlikely that significant rates of nucleotide synthesis could take place in the cell by a reversal of the degradative pathway in *E. coli*.

Phosphodeoxyribomutase Activity in Rabbit Tissues

Several initial experiments have suggested that rabbit tissues might also contain a distinctive phosphodeoxyribomutase. It has been assumed that the well-characterized phosphoglucomutase (from rabbit muscle) serves as a general mutase for all hexose- and pentose phosphates. A survey of rabbit tissues has disclosed that the glucomutase and deoxyribomutase activities do not parallel each other. In addition, there is not a constant ratio of these two activities during a 20-fold purification of the deoxyribomutase from rabbit liver. Finally, although the activities parallel each other in sucrose gradients (indicating a similar molecular size), they exhibit different chromatographic and kinetic properties.

The reaction requirements for rabbit liver deoxyribomutase are also somewhat different from the requirements for the *E. coli* enzyme. The rabbit liver enzyme is stimulated by Mg^{++} (whereas the *E. coli* enzyme is not), and is less active in the absence of glucose-1,6-diphosphate.

Physiological Studies

An attempt was made to find a gratuitous inducer for the group of enzymes involved in nucleoside degradation. Our earlier work had shown that the pentose-5-phosphates serve as the proximal intracellular inducers for these enzymes. We received from Dr. Roland Robins a group of nucleo-

side analogs containing modified pentose moieties, which, it was hoped, would be metabolized to non-degradable pentose-5-phosphate analogs. Unfortunately, none of the available analogs was effective as an inducer.

Future Plans

The immediate objective is to detect and characterize the polynucleotide deaminase enzyme, preferably from strains of *E. coli* which are low in ribonuclease activity. Initially, it would be useful to determine whether the inosinic acid content of *E. coli* t-RNA changes with nutritional variations, during relaxed synthesis of t-RNA or during bacteriophage infection. Such conditions might be more suitable for preparation and purification of the enzyme.

The provision of a suitable aminated substrate for the enzyme may be particularly difficult. A major corollary of the "wobble" hypothesis is that anticodons with adenylic acid at the 5'-terminus should not normally be found in nature. No exceptions to this supposition have yet been found in any of the known complete sequences of t-RNA.

Accordingly, it may be necessary to search for the enzyme with several types of substrate: (1) synthetic ribopolynucleotides and polynucleotide mixtures, containing labeled adenylic acid. Some of these mixtures, such as poly (AU + U) could provide looped regions approximating the size and shape of the anticodon loop of t-RNA. (2) Deaminase preparations should also be tested with heterologous t-RNA, since the enzyme may function in a manner analogous to that of the t-RNA methylases, and show no activity with homologous t-RNA. (3) The most useful substrate would be the presumptive natural one for the enzyme, *viz.* the products of RNA polymerase action on t-RNA genes. The success of this approach would require purification of the genes for t-RNA from some available source of DNA. Fortunately, this may be feasible in the case of the DNA from *Bacillus subtilis*, in which 80% or more of the t-RNA genes are clustured very near to the origin of the chromosome. Drs. Yoshikawa and Haas have isolated an initiation mutant of *B. subtilis* which carries out preferential multifork replications of the chromosome origin, and also excretes large amounts of DNA into the medium. The excreted

DNA has been tested and found to be enriched 5-fold for a genetic marker (ade-6) located distal to the t-RNA gene cluster. We thus expect that such DNA preparations are enriched by at least 5-fold for t-RNA genes.

We will attempt the additional purification of t-RNA genes from *B. subtilis* by hybridization of the excreted DNA with t-RNA, followed by chromatographic separation of the hybrids on MAK or hydroxyapatite columns, and ultimate enzymic removal of the paired t-RNA. If such a purification can be achieved, it should be possible to synthesize *in vitro* a general RNA substrate for most of the enzymes which modify to t-RNA.

STUDIES ON ERYTHROPOIESIS

(H. Borsook, B. Tattrie, K. Ratner, H. Pang, B. Stanfield)

General objectives are a) search for the factors influencing differentiation and development in erythropoiesis, b) analysis of the biochemical changes in erythroblasts from their earliest to mature stages cytologically and c) examination of what the interrelations are between the biochemical and cytological changes.

Erythropoiesis is one of the rare fields of biological investigation where the findings have both practical and theoretical interest. They enter into medicine (hematology), embryology (differentiation and development) and physiology (regulation of red cell and granulocyte production and synthesis of specific proteins, e.g. hemoglobin, cytochrome, catalase, etc.).

Methods

All our experimental material is from rabbits. The following methods have been developed: a) Erythroblasts are segregated according to their stage of maturation on a bovine serum albumin density gradient. b) An antiserum to rabbit erythrocytes was produced in the goat by which early and late rabbit erythroblasts can be separated. A combination of a) and b) gives better and quicker results than either alone. c) Conditions were devised for incubating erythroblasts *in vitro* under conditions in which they develop and differentiate, suffer little degeneration, and respond to stimulatory and inhibitory factors as *in vivo*. d) A method of scoring an erythroblast population was devised for stating, in numbers, the stage of development, normal and abnormal features.

Principal Findings To Date

The terminal differentiation in erythropoiesis is, cytologically, the loss of cytoplasmic basophilia. The subsequent changes constitute development and are experimentally distinguishable from differentiation. Three agents have been found which cause premature differentiation *in vitro* and also accelerate development. They are, (i) a high concentration of erythropoietin, (ii) nitrogen mustard, and (iii) erythrocytes — intact cells or their lysate. Actinomycin D inhibits differentiation but is without effect on development *in vivo* and *in vitro*.

These results explain the quick response *in vivo* to a large dose of erythropoietin: the appearance in the blood of abnormally large cells more quickly than can be accounted for as coming from the stem cell compartment. The view is widely held that once an erythroblast is generated its subsequent development cannot be changed. The new findings show it to be incorrect. The above agents act on cells peripheral to the stem cell compartment, increasing or inhibiting differentiation or development. Erythropoietin also accelerates the generation of erythroblasts; it has, therefore, two sites of action.

Concomitant with the disappearance of the cytoplasmic basophilia there is a change in the pattern of hemoglobin synthesis. It becomes 85 — 90 per cent instead of 20 per cent of the total protein synthesis, and it is the major, whereas earlier it is an acidic minor, hemoglobin that is chiefly synthesized.

The antiserum to rabbit erythrocytes lysed erythrocytes, under our experimental conditions, according to the equation:

$$L = \frac{K \frac{(A - 3CL)^2}{C^4}}{1 + K \frac{(A - 3CL)^2}{C^4}}$$

L is the degree of lysis, A and C are initial concentrations of antibody and cells respectively; and K is an equilibrium constant. The equation was deduced from the data. It conforms to a reaction model requiring the attachment of two antibody molecules to four specific sites on the cell for it to be lysed.

When a marrow cell mixture was treated with the highest concentration of antiserum the remaining erythroblasts were preponderantly basophilic. With progressive dilution of the antiserum, the proportions of the acidophilic erythroblasts and erythrocytes increased. The acidophilic erythroblasts are immunologically like erythrocytes, the basophilic are immature in this respect, and the changeover begins at the time they are losing their cytoplasmic basophils are a little more susceptible to lysis by antiserum than are erythrocytes. The severe anemia induced the production of immunologically abnormal erythroblasts. The latter are abnormal also in that they are more prone than the normal to early loss of nucleus (skipped division) with the formation of abnormally large reticulocytes and erythrocytes.

Some antibody remains attached to erythroblasts which are not lysed. After the antibody is removed the erythroblasts rapidly enlarge, differentiate and develop. Comparable cells not treated with antiserum under the same conditions show little or no development. These findings suggest that attachment of the antibody, even where there is no lysis, induces an irreversible change in the cell membrane and recalls the stimulation of development of the unfertilized egg by parthenogenetic agents.

Future Plans

1. Search for isolation of the stem cell compartment with the *in vitro* methods now in hand.
2. Investigation of the effect *in vitro* of the major hormones, e.g. thyroxine, growth hormone, sex hormones, etc.
3. Exploration among mutagenic agents like nitrogen mustard for their effects *in vitro* on differentiation and development.
4. Comparison of the relative rates of synthesis of hemoglobin — major and minors, heme and globin, cytochrome and catalase in early and late erythroblasts, the effects of prior treatment with antiserum, and the differences between normal and abnormal erythroblasts.

Reference

Effect of erythropoietin *in vitro* which simulates that of a massive dose *in vivo*. Borsook, H., K. Ratner, B. Tattrie, D. Teigler. *Nature, 217,* 1024 (1968).

ON THE REGULATION OF THE INITIATION OF DNA REPLICATION IN BACTERIA

(H. Yoshikawa, M. Haas, B. Jansen, E. Cook, J. Cerwin)

Evidence has accumulated that initiation of chromosomal replication plays a key role for the regulation of DNA replication in microorganisms. We find that *Bacillus subtilis* is one of the most suitable organisms for investigation of this type. We have reported the existence of protein synthesis prior to initiation of DNA synthesis during spore germination, a covalent linkage between newly synthesized DNA strands and termini of parental DNA strands, and configuration of a completed chromosome in spores. The present report concerns experiments that show how initiation of DNA replication is regulated in *B. subtilis*.

Simultaneous Initiation of Chromosomal Replication and Prophage Induction

As previously reported by us DNA synthesis inhibitors, 5-bromodeoxyuridine (5BUdR), Mitomycin C (MC) and thymine starvation, caused abnormal initiation of chromosomal replication. These inhibitors also induce prophages, suggesting that initiation of replication may be regulated by a mechanism similar to that causing prophage induction. If so, one should expect to observe abnormal initiations of replication simultaneously with prophage induction when lysogenic cells are treated with inhibitors of DNA synthesis.

B. subtilis lysogenic to either SPO2 or PBSH was treated with MC or ultraviolet light. Cellular DNA was isolated during the induction period and marker frequency was measured. In all cases ade-16/met ratio, an index of reinitiation, increased during prophage development, indicating that initiation of chromosomal replication was induced simultaneously with prophage development.

A Temperature-Sensitive Initiation Mutant

The above result suggested that initiation of replication is regulated by a mechanism similar to that causing prophage induction. If so, it should be possible to isolate bacterial mutants similar to temperature-sensitive inducible mutants of temperate phages. Such a mutant was found which induced abnormal initiation of chromosomal replication and PBSH phage at elevated temperatures.

A strain of *B. subtilis* which harbors defective prophage PBSH was treated with nitrosoguanidine and mutants which lysed when culture temperature was increased were selected. A mutant was isolated which grew normally only at temperatures lower than 35°. When growing cultures of this cell were shifted up to 45° lysis occurred in about one hour. Defective prophage PBSH was found in the lysate. Numbers of chromosomal forks were measured by marker frequency analyses. The mutant DNA had an ade-16/met ratio almost twice that of wild type DNA. Moreover, these ratios were independent of culture temperature. This is logical if it is assumed that synthesis rates of various cellular components are uniformly affected by temperature changes. However, the constant but abnormally high ade-16/met ratio for the mutant DNA was unexpected and needed further explanation.

The recent report of spontaneous transformation by a *B. subtilis* mutant led us to search for DNA ejected by the temperature-sensitive mutant. Significant amounts of ade-16 transforming activity were found in supernatant fractions from exponential cultures. Marker frequency analyses indicated that this mutant ejects substantial numbers of chromosomal fragments especially from areas near chromosomal origin. As the temperature was increased, increasing numbers of fragments were expelled indicating more initiations at higher temperatures.

These results suggest that regulation of chromosomal initiation is temperature-sensitive in this mutant. A logical consequence is that the initiation is regulated by repressor-derepressor type negative control mechanism.

Role of the Defective Prophage in Initiation of Chromosomal Replication

As stated above, a phage PBSH was induced by MC treatment simultaneously with a many-fold increase of ade-16/met ratio. A study of change in molecular weight of cellular DNA during the induction period showed that multiforked reinitiation at the chromosomal origin occurred due to the inhibitory action of MC. Furthermore, there was no indication of phage DNA replication. Phage DNA was produced by the fragmentation of cellular DNA. This indicated that phage PBSH was a replication-defective phage. The location of the phage gene on the host chromosome is not known. It is possible that the phage gene is located at the chromosomal origin and plays a regulatory role in initiation of chromosomal replication.

A Model of Regulation of Chromosomal Replication

Jacob and Brenner's "replicon hypothesis" proposed initiator protein as a regulatory material for initiation of chromosomal replication. At the end of each replication cycle, initiator is newly synthesized, or reaches a critical concentration to reinitiate a new round of replication. This regulation is a positive control in contrast to repressor-derepressor type negative control. Our experimental evidence contradicts this positive control mechanism, and suggests that the first step of initiation is regulated by a repressor-derepressor type negative control mechanism. Derepressor may be accumulated normally during each replication cycle or abnormally due to the inhibition of DNA synthesis.

Two models are possible for the action of the derepressor. The first is that derepressor will separate repressor from a structural gene which codes for the initiator protein. The initiator thus synthesized may in turn initiate chromosomal replication. The second model does not require the synthesis of initiator protein. Release of the repressor may automatically induce replication at the origin of the chromosome. This model imposes a specific DNA structure at the origin of the chromosomes which is normally insensitive to DNA polymerase action due to repressor binding.

PBSH DNA showed a peculiar sedimentation behavior in different salt concentrations indicating that the DNA is more flexible than normal double-stranded DNA. Moreover, renaturation kinetics of ade-16 marker of the PBSH DNA showed that the DNA contained clusters of similar base sequences. This evidence supports the hypothesis that the phage gene is located at the host chromosomal origin and plays a role in negative control of initiation of chromosomal replication.

Future Plans

1. *Structure of initiation region of the <u>B. subtilis</u> chromosome*

Preliminary experiments showed that short 5-BUdR-containing DNA fragments were synthesized very soon after the onset of DNA synthesis by germinating spores. These short strands seem to be covalently linked to pre-existing DNA.

We are now trying to reproduce this result without using BUdR to avoid BUdR's toxic effects. To obtain short pulses of less than 30 sec, with H^3-TdR, a new method of stopping DNA replication in the spore should be found. It has not been successful so far. If we succeed in obtaining short DNA fragments from thymidine DNA strand, we will label both 5-BUdR and thymine DNA with P^{32}. Base analysis of the P^{32} DNA labeled for various times should give an average base composition of the starting end of the *B. subtilis* chromosome.

The other line of study will be purification of ade-16 gene and the analysis of its structure. The denaturation and renaturation of ade-16 gene of PBSH phage DNA indicated the existence of a unique nucleotide sequence in or near this gene. The sequence is such that it markedly accelerates condensation of two complementary strands during renaturation.

In practice, this method provided the means to purify ade-16 gene. We will continue the effort to purify ade-16 gene by repeated denaturation-renaturation treatment. If the gene is sufficiently purified, the analysis of base component and base sequence analysis (such as purine, pyrimidine clusters and C and T cluster, etc.), will be made on the DNA. This work should clarify not only observed peculiar behavior of ade-16 gene in renaturation, but also the unique structure at the origin of *B. subtilis* chromosome.

2. *Temperature-sensitive inducible mutants*

Efforts will be made to characterize the temperature-sensitive mutant which makes DNA higher in ade-16/met ratio in exponential phase at low temperature, excrete DNA to the medium at high temperature and finally lysed at 45° producing PBSH.

Preliminary experiments showed that the lysis at high temperature is chloramphenicol resistant. This eliminates the PBSH induction as the possible cause of the lysis and suggests that the mutation might involve in the alteration of membrane structure. Such a change may affect the control of the initiation of DNA.

We will pursue to identify the mutation in this mutant from this point of view. We will also try to isolate many more mutants similar to this mutant to make genetic analysis of such mutations.

DNA REPLICATION *IN VIVO* BY A TEMPERATURE-SENSITIVE POLYNUCLEOTIDE LIGASE MUTANT OF T4

(J. Hosoda, E. Mathews, and S. Stephenson)

Okazaki et al. have proposed a mechanism of *in vivo* DNA replication in which short single-stranded segments of both DNA strands are synthesized and subsequently joined by a polynucleotide ligase type of reaction to form long intact strands. It was reported that amber mutants in ligase genes of T4D bacteriophage cannot continue DNA synthesis under restrictive conditions, and that DNA synthesized was found as short fragments following denaturation. We found additional evidence that polynucleotide ligase plays an essential role in DNA replication and supports the mechanism proposed by Okazaki et al.

A mutant of T4D, tsA80, which synthesizes a temperature-sensitive ligase, could not continue DNA replication at high temperatures. DNA strands synthesized by tsA80 at high temperatures accumulated as short fragments in a single peak of about 16S when analyzed in alkaline sucrose gradients. These DNA fragments served as precursors of long DNA strands when the temperature of the culture was subsequently lowered to enable the activity of temperature-sensitive phage ligase to resume. The short fragments of DNA made at 42° were not covalently connected to the bulk DNA previously made at 25°. Although our experiments are not a complete proof of all aspects of the theory, the results seem to agree with Okazaki's model, in which DNA replication *in vivo* takes place discontinuously on both strands with the newly made portion of DNA temporarily existing as short fragments that are subsequently joined and connected by the action of ligase to the long strand of previously synthesized DNA. In our experiments, the short fragments of DNA made in the absence of ligase action were not covalently linked to the bulk DNA strands, but this, as well as the presence of short strands of pulse-labeled DNA in various organisms reported by Okazaki, does not exclude the possibility that the short strands are produced by the action of an endonuclease on the newly made portion of DNA, which is in a different physical state from that made earlier and hence may be more susceptible to the enzyme. Although the fragments of DNA accumulated by tsA80 at high temperature serve as precursors of long DNA strands, this does not necessarily mean that they are intermediate precursors in normal

replication. An amber mutant, amH39X, of the ligase gene accumulated newly synthesized DNA as fragments of the same size as tsA80 in the corresponding condition. In the case of the amber mutant, a detailed analysis at low temperature indicated that the apparently homogeneous peak contained a mixture of DNA segments, a portion of which has reached a considerable length and which is then probably broken by endonuclease. If the DNA accumulated by tsA80 is also a mixture corresponding to that found with amH39X, all components served as precursor material.

Although the failure of joining and repairing by ligase mutants of T4 *in vivo* indicates that the host ligase cannot replace phage ligase, some participation of host enzyme(s) was apparent since leakiness of the ligase mutants depended on the host used and the condition of the culture. Gene 46 mutation prevented the joining of DNA fragments accumulated by tsA80 at high temperatures when the ligase activity was restored. The function of gene 46 is not required for joining of the normal precursor segments of DNA. One possible explanation for this inhibition is that if the gene 46 mutation is related to the exonuclease III type function, accumulation of 3'-phosphate ends might have taken place during prolonged incubation and prevented joining or elongation of the strands.

Future Plans

In a collaborative study (J. Hosoda and W. Szybalski, unpublished), we have analyzed the ability of the various newly synthesized fragments of ^{3}H-DNA produced in *E. coli* cells infected with T4amH39 or T4tsA80 and isolated by alkaline sucrose gradient centrifugation, to hybridize with the separated H and L strands of T4 DNA. Our experiments show that one particular fraction (16S) of these newly-synthesized fragments exhibits a marked preference for hybridization with the H strand (H to L ratios up to 3:1), which result indicates that it was preferentially copied from strand H, which is inactive in early T4 transcription.

We plan to expand these hybridization experiments to reveal the mechanism of DNA replication in absence of ligase activity.

STRUCTURE AND FUNCTION OF BACTERIAL RIBOSOMES

(K. Hosokawa, M. A. Q. Siddiqui, and John L. Prehn)

The establishment of a method for reconstituting functionally active ribosomes from subparticles and proteins (K. Hosokawa et al., *PNAS, 55*: 198, 1966; T. Staehelin and M. Meselson, *JMB, 16*: 249, 1966) has contributed to the knowledge of the mechanism of polypeptide synthesis and provides an approach to studying the internal structure and function of ribosomes.

Ribosomes of *Escherichia coli* consist of 30S and 50S subunits. The structure and function of 50S subunits, which have been less explored than those of 30S subunits, was examined by studying the role of 5S ribosomal RNA (rRNA) which is associated with 50S subunits, in polypeptide synthesis.

Physicochemical Studies of 5S Ribosomal RNA

We have devised a simple and fast method for the purification of 5S rRNA. Sedimentation constant of purified 5S rRNA was 4.65.

From the base sequence of 5S rRNA, a complex tertiary structure was suggested (Brownlee et al., *Nature, 215*: 735, 1967; Cantor, C. R., *PNAS, 59*: 476, 1968; Boedtker and Kelling, *BBRC, 59*: 758, 1967). Such a compact configuration of 5S rRNA, like that of tRNA, should be comparatively resistant to the action of RNase. But 5S rRNA was more sensitive to RNase than were the tRNAs or 16S and 23S rRNA at 10^{-3} M Mg^{++}. By increasing the concentration of Mg^{++} from 3×10^{-4} M to 10^{-2} M, tRNA became more resistant against RNase treatment, but the RNase sensitivity of 5S rRNA and that of 16S and 23S rRNAs remained unchanged. When the temperature of RNase treatment was lowered down to 23°(or 4°), 5S rRNA showed increased resistance to pancreatic RNase as did tRNA when the concentration of Mg^{++} was increased, although the rate of degradation of RNA decreased. There were no substantial differences in the sedimentation profiles of 5S rRNA in the presence or absence of Mg^{++}.

Dissociation of 5S RNA from 50S Ribosomal Subunit

RNA phenol-extracted from crude 50S ribosomal subunits gave the ratio of tRNA: 5S rRNA:23S rRNA = 1-2:1:1 as calculated from the optical pattern of elution on MAK column. Washing the 50S subunits in TMA (-4) resulted in

complete removal of tRNA but 5S rRNA still remained associated with 50S ribosomal subunits in 1.2:1 molar ratio. RNA extracted from purified 30S ribosomal subunits did not contain 5S rRNA.

40S CsCl core particles (ρ = 1.65) derived from 50S subunits by CsCl centrifugation retain 5S rRNA in 1:1 molar ratio (per 23S rRNA). Dense CsCl core particles (ρ = 1.71) made by subjecting 50S subunits to CsCl centriguation in the presence of citrate and Mg^{++} still retained 5S rRNA in one mole per mole particle. When Mg^{++} was reduced from 0.04 M to 0.02 M in CsCl solution, a partial loss of 5S rRNA from the dense core was observed (Table 1) with concomitant decrease of the yield of dense particles presumably from breakage. In the absence of Mg^{++}, the ribosome structure was completely broken down in CsCl with chelating agents.

A milder condition for dissociating 5S rRNA was produced by dialysis of 50S subunits vs Mg^{++}-free buffer containing 0.5 M NH_4Cl. The ribonucleoprotein particles (50S-A) retained only 20% of 5S rRNA. The sedimentation constant of 50S-A particle was 44S. Treatment of 50S-A particles with 0.5 µg per ml pancreatic RNase for 10 min at 4° resulted in complete breakdown of RNA as shown by MAK-column chromatography, but the RNA of native 50S subunit was intact after the same treatment. Polyacrylamide gel electrophoresis of protein from 50S-A particles showed that a single protein band closest to the origin was missing (Siddiqui and Hosokawa, *BBRC, 32*: 1, 1968). By short dialysis vs Mg^{++}-free buffer containing 0.5 M NH_4CL and chelating agent, 50S subunit was converted to 40S particles which were completely free from 5S rRNA but retained all protein components as analyzed by polyacrylamide gel electrophoresis.

It is concluded that 5S rRNA is tightly associated with 50S ribosomal subunits in the presence of Mg^{++}. Dialysis of 50S subunits against Mg^{++}-free buffer containing 0.5 M NH_4CL and chelating agent resulted in complete removal of 5S rRNA with all the protein components present. The particle was converted to 40S and rendered remarkably sensitive to RNase suggesting a partially uncoiled structure which was not converted to 50S particles even when excess 5S rRNA was added.

Release of 5S rRNA and splitting of proteins from 50S subunits seems to proceed independently. Removal of Mg^{++} or treatment with chelating reagents caused unfolding of the structure of ribosomes or the subparticles

and release of 5S rRNA. On the other hand, treatment with high concentrations of salts such as CsCl resulted in preferential dissociation of proteins from 50S ribosomes.

Treatment of 50S ribosomal particles with sodium citrate in the presence of Mg^{++} caused progressive conformational change without loss of 5S rRNA or protein components. Although 5S rRNA was associated with the particles, exchange with externally added ^{32}P-labeled 5S rRNA could be observed. ^{32}P-tRNA did not bind to the particle in the same condition. ^{32}P-5S rRNA bound to the particles was diluted by the subsequent addition of excess amount of cold 5S rRNA but not by tRNA. Dilution of bound ^{32}P-5S rRNA was also affected by adding 5S rRNA from *Pseudomonas* and *Azotobacter* to the same extent.

EVOLUTION OF HYDROXYLASES OF AROMATIC COMPOUNDS IN *PSEUDOMONAS*

Structure and Function of Hydroxylases

(K. Hosokawa and B. Hesp, in collaboration with Department of Chemical Biodynamics)

B-hydroxybenzoate 3-hydroxylase and salicylate 1-hydroxylase are inducibly synthesized by *Pseudomonas putida* M6, a soil microorganism, and catalyze the following reactions:

$$\text{p-hydroxybenzoic acid} + \text{NADPH} + H^+ + O_2 \rightarrow \text{Protocatechuic acid} + NADP^+ + H_2O \quad (1)$$

$$\text{Salicylic acid} + \text{NADH} + H^+ + O_2 \rightarrow \text{catechol} + CO_2 + NAD^+ + H_2O \quad (2)$$

The two hydroxylases have many similar properties (1) Flavin adenine dinucleotide bound protein, (2) Requirement for reduced pyridine nucleotide, (3) pH optima are 7.5 (phosphate buffer) and 8.0 (Tris buffer) for both, (4) Monooxygenase, (5) Molecular weight ranges between 70,000 and 90,000. The enzyme was crystallized (Hosokawa and Stanier, *JBC, 241*: 2453, 1966). Under anaerobic conditions, the enzyme-bound FAD was reduced by NADPH dependent on the presence of p-hydroxybenzoate. Solutions of p-hydroxybenzoate hydroxylase show two absorption maxima at 375 mμ and 450 mμ due to bound FAD. When substrate is added to the enzyme, the 450 mμ peak sharpens and the 375 mμ peak shifts toward longer wavelength. P-hydroxybenzoate might

influence the physicochemical nature of enzyme-bound FAD. This idea was confirmed by circular dichroism measurement of the enzyme solution. There was a negative signal with a maximum at 455 mμ and a positive signal with a maximum at 370 mμ. When the substrate is added, a remarkable change occurs. The positive signal shifts toward longer wavelength, the negative signal is generally reduced by approximately 20% and the peak shape generally broadens. These results strongly support that the substrate-enzyme complex is formed before the hydroxylation reaction takes place.

Control of Synthesis of P-Hydroxybenzoate Pathway Enzymes

P-hydroxybenzoate is converted to β-ketoadipate by means of five inducible enzymes: p-hydroxybenzoate 3-hydroxylase, protocatechuate 3,4-oxygenase, β-carboxy cis,cis-muconate lactonizing enzyme, carboxymuconolactone decarboxylase, and β-ketoadipate enol-lactone hydrolase. The mode of synthesis of the first two enzymes was studied and compared with that of the last three enzymes. P-hydroxybenzoate 3-hydroxylase is induced specifically by p-hydroxybenzoate, but not by others. Protocatechuate 3,4-oxygenase is induced by protocatechuate as well as by p-hydroxybenzoate as shown by mutants lacking p-hydroxybenzoate 3-hydroxylase. The operon of the hydroxylase is thought to be independent from that of the oxygenase. The repressor for the hydroxylase operon responds to p-hydroxybenzoate and the repressor for the oxygenase operon to both p-hydroxybenzoate and protocatechuate.

Future Plans

Bacterial ribosomes

1. *Role of 5S ribosomal RNA in polypeptide synthesis.* By preparing 50S ribosomal subunits completely free from 5S ribosomal RNA, the functions of the 5S-rRNA-free subunits will be examined and the effect of 5S rRNA on the restoration of defective functions will be studied.

TABLE I

PRESENCE OF 5S rRNA IN VARIOUS RIBONUCLEOPROTEIN PARTICLES DERIVED FROM 50S RIBOSOMES OF *E. Coli*

50S ribosomes or its subparticles	Concentration of Mg^{++} treated	Ratio: 5S rRNA/23S rRNA	
		% in A_{260}	molar * ratio
50S ribosomal particles	$3 \times 10^{-4} - 10^{-2}$ M	5.1	1.4
CsCl core particles (ρ = 1.65)	4×10^{-2} M	4.2	1.2
Dense CsCl core particles (ρ = 1.71)	4×10^{-2} M	4.3	1.2
	2×10^{-2} M	2.5	0.7
50S-A (44S particles)	0	1.1	0.3
30S ribosomal particles	$3 \times 10^{-4} - 10^{-2}$ M	0.0	0.0

RNA extracted from 50S ribosomes or their derivatives was subjected to MAK column chromatography. Total A_{260} units for 5S rRNA and 23S rRNA were calculated from the optical pattern of elution.

*Calculations were made assuming that molecular weights for 23S rRNA and 5S rRNA were 1.1×10^{-6} and 3.9×10^{4} respectively.

2. *Studies of the action of antibiotics which inhibit ribosomal function.* Neomycin is effective at a lower concentration than streptomycin and induces coding changes which are clearer and somewhat different from those caused by streptomycin. Localization of the neomycin site on 30S ribosomal subunits will be studied.

   Tetracyclines inhibit protein synthesis by acting on 50S or larger subunits of ribosomes. The mechanism of action of these antibiotics will be studied using reconstitution technique.

Evolution of hydroxylases of Pseudomonas

Similarities in properties and functions of p-hydroxybenzoate and salicylate hydroxylases suggest that they have the same evolutionary origin. Comparison of the structure and function of these hydroxylases is in progress. Comparative analyses of the mode of synthesis of p-hydroxybenzoate and salicylate hydroxylases will be made to explore the evolution of genetic control systems.

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